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Review

Mitochondrial shaping cuts

Mafalda Escobar-Henriques *, Thomas Langer

Institute of Genetics and Center for Molecular Medicine (CMMC), University of Cologne, Cologne, Germany

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Abstract

A broad range of cellular processes are regulated by proteolytic events. Proteolysis has now also been established to control mitochondrial morphology which results from the balanced action of fusion and fission. Two out of three known core components of the mitochondrial fusion machinery are under proteolytic control. The GTPase Fzo1 in the outer membrane of mitochondria is degraded along two independent proteolytic pathways. One controls mitochondrial fusion in vegetatively growing cells, the other one acts upon mating factor-induced cell cycle arrest. Fusion also depends on proteolytic processing of the GTPase Mgm1 by the rhomboid protease Pcp1 in the inner membrane of mitochondria. Functional links of AAA proteases or other proteolytic components to mitochondrial dynamics are just emerging. This review summarises the current understanding of regulatory roles of proteolytic processes for mitochondrial plasticity.

Keywords: Mitochondria; Proteolysis; Fusion; AAA proteases; Rhomboid; Ubiquitin-proteasome

1. Introduction

Mitochondria are dynamic organelles whose structure depends on the cell type and physiological conditions and their constant morphological adaptations rely on fusion and fission events [1]. Apoptosis, inheritance of mitochondrial DNA, defence against oxidative stress or development through spermatogenesis are examples of cellular processes dependent on a correct balance between mitochondrial fusion and fission [2]. An essential role for the maintenance of mitochondrial activities has been assigned to proteases within mitochondria, many of which are ubiquitously distributed in eukaryotic cells and highly conserved from bacteria to man [3]. Energy-dependent proteases present in the inner membrane or the matrix space exert key functions within mitochondria. They do not only conduct the surveillance of protein quality control by completely degrading misfolded proteins to peptides but also regulate mitochondrial biogenesis by processing and thereby activating mitochondrial proteins. Moreover, the cytosolic ubiquitin-proteasome system was found to be functionally linked to mitochondria. It mediates the turnover of nuclearly encoded mitochondrial preproteins prior to their import into the organelle [4] and affects, at least under certain conditions, the stability of mitochondrial outer membrane proteins [5].

Increasing evidence suggests that mitochondrial plasticity, and in particular mitochondrial fusion, is controlled by proteolysis at multiple steps. Mitochondrial fusion is unique, as two membranes have to simultaneously fuse in order to maintain the organelle integrity [6]. A central role in mitochondrial fusion processes has been assigned to a complex containing the conserved GTPases Fzo1 (or mitofusin in mammals) and Mgm1 (or Opa1 in mammals) and Ugo1 (in yeast) [7]. Both Fzo1 and Mgm1 were recently found to be controlled by proteolysis. Moreover, a number of proteases and assisting proteolytic proteins were recently found to be required for the maintenance of mitochondrial morphology [8,9]. Thus, although the molecular basis of these recent findings remains to be elucidated, additional regulatory roles of proteolysis for mitochondrial dynamics may emerge.

This review will focus on proteolysis-associated events connected to mitochondrial dynamics, whereas our current understanding of basic functions of the proteolytic systems involved has been reviewed elsewhere [3,10,11].

* Corresponding author. Mailing address: Institut für Genetik, Universität zu Köln, Zülpicher Str. 47, 50674 Köln, Germany. Tel.: +49 221 470 4856; fax: +49 221 470 6749.
E-mail address: Mafalda.Escobar@uni-koeln.de (M. Escobar-Henriques).

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2. The rhomboid protease in the inner membrane and Mgm1/Opa1 processing

The dynamin-like GTPase Mgm1 is a conserved protein essential for mitochondrial fusion in *S. cerevisiae* [1]. The absence of Mgm1 leads to the accumulation of fragmented mitochondria, to a loss of mitochondrial DNA and therefore to a petite phenotype [12–15]. Mgm1 is present in two isoforms within mitochondria, a long form of 97 kDa (l-Mgm1) anchored to the inner membrane and a short form (s-Mgm1) only loosely associated with the inner or outer membrane [13,15,16] (Fig. 1). It is conceivable though only speculative at present that the different localization of both Mgm1 isoforms contributes to the coordinated fusion of the outer and inner mitochondrial membranes. Mgm1, specially l-Mgm1, interacts through Ugo1 with Fzo1 [17], all three proteins being essential for mitochondrial fusion (Fig. 1). Only about 10% of these proteins can be found co-immunoprecipitated in a complex that appears rather dynamic or instable [13,16], which could account for timing and coordinated mitochondrial fusion.

The protease Pcp1 in the inner membrane cleaves Mgm1 in its amino terminal part thereby generating s-Mgm1 [18–20]. Such cleavage occurs after removal of the mitochondrial targeting sequence of Mgm1 by the mitochondrial processing peptidase MPP. Interestingly, Mgm1 cleavage by Pcp1 was found to depend on cellular ATP levels [21]. It may therefore function as an energetic sensor regulating mitochondrial fusion through the balanced formation of both Mgm1 isoforms upon import of this nuclear-encoded pre-protein (Fig. 1). According to the alternative topogenesis model [21], an amino terminal hydrophobic segment serves as a stop-transfer signal at low ATP preventing membrane insertion of a subsequent hydrophobic segment harbouring the Pcp1 cleavage site. As a consequence, only l-Mgm1 is formed which remains anchored to the inner membrane. At high ATP, however, Mgm1 can be pulled further into the matrix by the ATP-dependent mitochondrial import motor. The second hydrophobic segment is inserted into the inner membrane and cleavage by Pcp1 results in the generation of s-Mgm1 [21]. Although both isoforms would only be formed during import according to this model, Pcp1 mediated processing of the pre-existing long form of Mgm1 cannot be excluded at present.

Mitochondrial fusion depends on both s-Mgm1 and l-Mgm1 [18] and therefore it is not surprising that Δ*pcp1* cells harbouring only unprocessed Mgm1 present a mitochondrial morphology defect [9]. Expression of the s-Mgm1 in Δ*pcp1* cells restores at least partially the respiratory competence of the cells and normal mitochondrial morphology [18]. These findings indicate that impaired Mgm1 processing accounts for most of the deficiencies of Δ*pcp1* cells. Nevertheless, other substrates of Pcp1 do exist within mitochondria. Another Pcp1 substrate is represented by cytochrome *c* peroxidase [22], Ccp1, a nuclear encoded scavenger protein for reactive oxygen species, which is targeted to the intermembrane space by a bipartite presequence. Maturation of Ccp1 occurs in the inner membrane by the sequential action of the ATP-dependent *m*-AAA protease and Pcp1 [22].

Pcp1 is the mitochondrial member of rhomboid proteases which comprise a highly conserved protein family [23,24]. These proteases are integral membrane serine endoproteases and generally clip substrate proteins within their membrane-spanning segment [25,26]. They are therefore referred to as intramembrane cleaving proteases [27] although it is still an open question whether they indeed cleave substrates in membrane-embedded parts. Rhomboid homologues with putative mitochondrial targeting sequences exist from yeast to humans [27]. PARL (presenilin-associated rhomboid-like), a human Pcp1 homologue, was able to process both Ccp1 and Mgm1 in yeast, pointing to an evolutionary conserved role of the mitochondrial rhomboid protease [19]. Mutations in OPA1, the human homologue of Mgm1, are the cause for the most common form of hereditary blindness, the autosomal-dominant optic atrophy (ADOA), which is characterized by a progressive degeneration

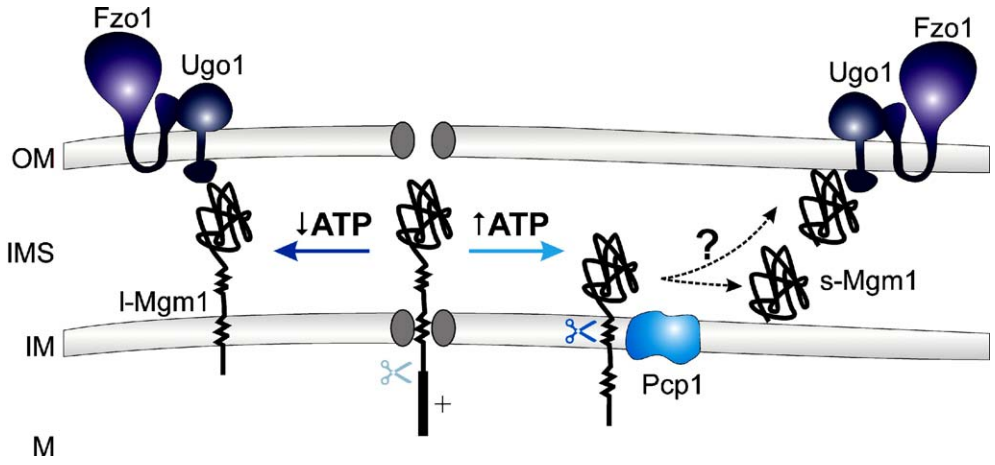

Fig. 1. Mgm1 processing in the inner membrane of mitochondria. A complex containing Mgm1, Ugo1 and Fzo1 forms the fusion machinery in the outer membrane of mitochondria. Mgm1 is present in two forms, a long form (l-Mgm1), which is integrated into the inner membrane by the first amino terminal hydrophobic segment and a short form (s-Mgm1), which is loosely associated with the inner or outer membrane. In the presence of high ATP levels (↑ATP), Mgm1 is further imported leading to the insertion of the second hydrophobic stretch containing a cleavage site for the rhomboid protease Pcp1. IM, inner membrane; IMS, inter membrane space; M, matrix and OM, outer membrane.

of the optic nerve and loss of visual activity [28–31]. Mitochondrial fusion defects due to impairment of proteolytic processing of Opa1 might be at the basis of this pathogenesis. It should be emphasized, however, that a functional conservation of Mgm1/Opa1 processing by a rhomboid protease remains to be demonstrated. Opa1 is present in several isoforms in various tissues [32], but direct evidence for an involvement of the mammalian Pcp1 homologue PARL has not been provided. Even more, it is presently not clear whether isoforms of Opa1 are exclusively generated by alternative splicing [32] or whether proteolytic cleavage is required.

3. Proteolytic regulation of Fzo1 in the outer membrane of mitochondria

The GTPase Fzo1 represents yet another component of the mitochondrial fusion machinery which is under proteolytic control. Mitochondrial fusion mediated by Fzo1 depends on a tight control of its steady state concentration, controlled by ongoing degradation in vegetatively growing yeast cells. Mechanisms guiding the turnover of mitochondrial outer membrane proteins are generally hardly understood. For the proteolytic breakdown of Fzo1, however, two independent proteolytic pathways are now emerging [5,33]: proteolysis can be either mediated by the ubiquitin-proteasome system (UPS) or by another, yet poorly described, proteolytic system in an UPS-independent manner (Fig. 2).

3.1. Fzo1 degradation by the ubiquitin-proteasome system

During the mating process, upon contact with its partner pheromone, yeast cells undergo a number of morphological changes, such as fragmentation of the mitochondrial network [33]. Fzo1 is an essential component for fusion and its absence leads to the accumulation of fragmented mitochondria [34–37]. Therefore, it is not completely surprising that the cell cycle arrest with the mating factor alpha, which leads to mitochondrial fragmentation, is accompanied by specific degradation of Fzo1 [33] by the UPS [5], the central proteolytic system in the cytosol of eukaryotic cells [10] (Fig. 2). In general, an unstable substrate is targeted for degradation by the covalent binding of ubiquitin, a small modifier protein, through a succession of three enzymatic steps. These are catalysed by an enzymatic cascade including E1-(ubiquitin-activating enzymes), E2-(ubiquitin-conjugating enzymes) and substrate-specific E3-enzymes (ubiquitin ligases) [10]. Fzo1 degradation in the presence of alpha factor is inhibited under restrictive conditions in cells harbouring a temperature-sensitive allele of the ubiquitin-activating enzyme Uba1 demonstrating the requirement of ubiquitin for proteolysis [5]. Similarly, proteolysis was inhibited in yeast strains harbouring mutant 26S proteasomes [5]. Other components of this proteolytic system and the mechanism of substrate dislocation from the outer membrane for proteolysis by 26S proteasomes are currently not known. These findings link the UPS to the degradation of Fzo1 and, concomitantly, to the fragmentation of mitochondria upon alpha factor induced cell cycle arrest. The physiological relevance of the proteolytic breakdown of Fzo1, however, remains to be elucidated as mitochondrial fragmentation per se is not essential for the mating process.

3.2. Mdm30-dependent degradation of Fzo1

The physiological importance of a defined concentration of Fzo1 is much better understood in vegetatively growing cells. A high throughput screening for components essential to maintain a normal mitochondrial morphology revealed that absence of the F-box protein Mdm30 leads to aggregated/fragmented mitochondria [9]. F-box proteins harbour a protein motif of approximately 50 amino acid residues that interacts with Skp1, a core component of the E3-SCF ubiquitin ligases (Skp1, Cdc53 and F-box) [38,39]. They thereby determine the substrate specificity of these E3 enzymes and ensure ubiquitin-dependent degradation by 26S proteasomes. Consistently, Fzo1 was found to be degraded in vegetatively growing cells in an Mdm30 and ATP

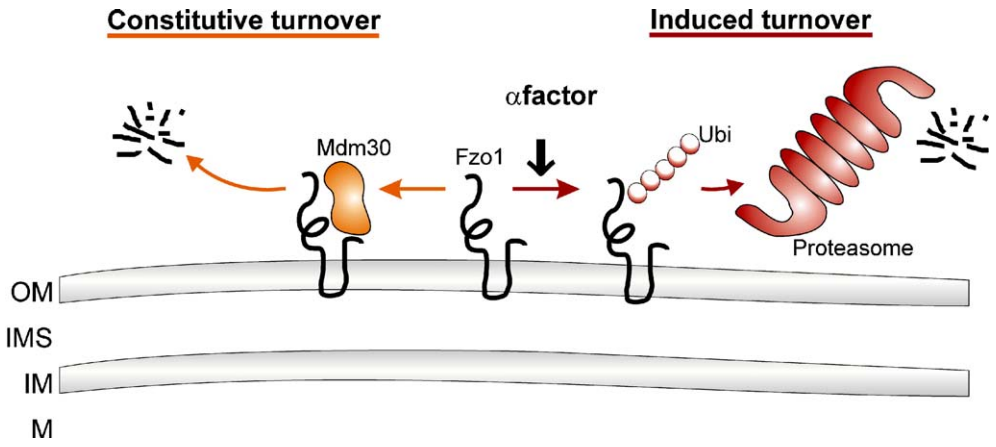

Fig. 2. Proteolysis of Fzo1 along two independent proteolytic pathways. Mitochondrial fusion depends on a tight control of the Fzo1 concentration in vegetatively growing cells. Under these conditions, Fzo1 is constitutively degraded through its association with the F-box protein Mdm30 (constitutive pathway represented in orange). On the other hand, Fzo1 is degraded by the UPS upon alpha factor-induced cell cycle arrest (induced pathway represented in red). IM, inner membrane; IMS, inter membrane space; M, matrix and OM, outer membrane.

dependent manner [5] (Fig. 2). Surprisingly, however, proteolysis was not impaired under restrictive conditions in yeast cells carrying temperature-sensitive alleles of the ubiquitin-activating enzyme Uba1, of core components of SCF complexes or of proteasomal subunits, i.e., is not mediated by the SCF or the UPS. On the other hand, UPS-dependent proteolysis of Fzo1 upon alpha factor induced cell cycle arrest does not depend on Mdm30. Thus, Mdm30 is not involved in the ubiquitin-mediated proteolysis of Fzo1 but rather is part of an alternative proteolytic pathway. This is reminiscent of an increasing number of F-box proteins which do not function as part of SCF ubiquitin ligases [40,41].

Deletion of *MDM30*, in contrast to any other known genes required for mitochondrial morphology, results in an increased steady state concentration of Fzo1, suggesting that Mdm30 affects fusion exclusively by regulating the stability of Fzo1 [5,42]. In agreement with this hypothesis, overexpression of Fzo1 in yeast wild type cells leads to mitochondrial fragmentation and mimics the phenotype of Δ*mdm30* cells [5]. Mitochondrial fragmentation and aggregation in Δ*mdm30* cells destabilizes mitochondrial DNA, resulting in the loss of respiratory competence (*petite* phenotype) [42].

These findings demonstrate that a tight regulation of Fzo1 levels in a cell is crucial for the maintenance of mitochondrial morphology. In analogy to mammalian mitofusin [43,44], it can be envisaged that mitochondria may tether in the presence of increased Fzo1 concentrations but cannot complete fusion, rationalizing the aggregation of mitochondria in Δ*mdm30* cells. One possibility could be that Mdm30 promotes Fzo1 degradation in connection to the fusion process allowing fusion to be completed.

The close link of Mdm30 to mitochondrial fusion is substantiated by a recently discovered novel phenotype of Δ*mdm30* cells. Mitochondria undergo extensive remodelling during sporulation and meiosis, dependent on fusion and fission events [45,46]. Mdm30 was shown to be involved in this process, since mitochondrial fusion is blocked during sporulation of Δ*mdm30* cells, leading to a mitochondrial inheritance defect in ascospores [47].

4. Additional roles of the UPS for mitochondrial morphology?

The studies on the stability of Fzo1 in yeast cells in presence of the alpha factor provide a direct link of the UPS to mitochondrial dynamics; however, additional functions of the UPS are likely. A role for ubiquitylation has been proposed in mammalian spermatogenesis, fertilization, and sperm quality control [48–50]. During spermatogenesis, still unknown targets on the mitochondrial surface are ubiquitinated. Then, upon fertilization and by encountering the egg's destruction machinery, sperm mitochondria undergo dramatic reduction. Ubiquitin tagging of mitochondria in sperm cells may serve as a death signal for paternal mitochondria during fertilization, thus strictly ensuring the maternal inheritance of mitochondrial DNA in mammals.

Ubiquitylation may also play a role for mitochondrial inheritance in yeast independent of its role for Fzo1 degradation. Overexpression of a mutant form of ubiquitin unable to form polyubiquitin chains on lysine 63 or a double deletion in the E2 enzymes Ubc4 and Ubc5 affects mitochondrial inheritance and induces mitochondrial aggregation [51]. Similarly, other E2 and E3 enzymes have been shown to interfere with mitochondrial morphology [8,9]. Yeast cells lacking Cdc53, a core component of SCF complexes, or the SCF-associated E2 enzyme Cdc34 present fragmented mitochondria [8]. This phenotype is also observed in *pre1* cells harbouring mutant 20 S proteasomes [8]. Notably, the appearance of mitochondria in these cells is strikingly different to Δ*mdm30* cells which accumulate aggregated mitochondria [9], substantiating the finding that Mdm30 acts independently of the UPS. Thus, additional regulatory roles of the UPS for mitochondrial dynamics appear to exist. This notion is also supported by the localisation of yeast deubiquitinase Ubp16 to the outer surface of mitochondria, although the functional relevance of this observation is presently not understood [52].

Additional E3 ubiquitin ligases affecting mitochondrial morphology include Rsp5 [51] and Not4 [9] and the F-box protein Mfb1 which localises to mitochondria [47]. Deletion of *MFB1* does not impair mitochondrial fusion but results in the formation of aggregated mitochondria [47]. The function of Mfb1 therefore appears to be distinct from Mdm30 and indeed it is not required for Fzo1 turnover [47]. Yeast mutant cells defective in the E3 enzymes Rsp5 and Not4 harbour fragmented and aggregated mitochondria [9,51]. Rsp5 is involved in many cellular processes including biosynthesis of unsaturated fatty acids and multivesicular body sorting [53] but a direct role for mitochondrial dynamics remains speculative. It is conceivable, though not demonstrated, that aberrant mitochondria in *rsp5* mutant reflect disturbances in the lipid metabolism due to a transcriptional block of the fatty acid desaturase Ole1 [54,55]. *OLE1* transcription is regulated by the activator Spt23 which is activated at the ER membrane by Rps5- and ubiquitin-dependent proteasomal processing [54]. The ubiquitin ligase Not4 [56] is a core component of the CCR4–NOT complex, which serves as a regulatory platform that senses and/or transmits nutrient and stress signals to various downstream effectors [57]. Accordingly, Not4 affects cellular processes as diverse as transcription repression and mRNA degradation and might therefore only indirectly affect mitochondrial morphology.

5. AAA proteases in the inner membrane affect mitochondrial morphology

The maintenance of the mitochondrial network depends also on proteolytic events in the inner membrane. In addition to the rhomboid protease Pcp1, increasing evidence suggests a role of AAA proteases for mitochondrial morphology. AAA proteases are built up of conserved subunits which assemble into large, presumably hexameric complexes in the inner membrane. All subunits harbour a metallopeptidase domain as well as an ATPase domain, characteristic of the AAA superfamily of ATPases. Two AAA proteases, which expose their catalytic sites to opposite membrane surfaces, are apparently ubiquitously present in the inner membrane (Fig. 3) [3,11,58]: the *i*-AAA

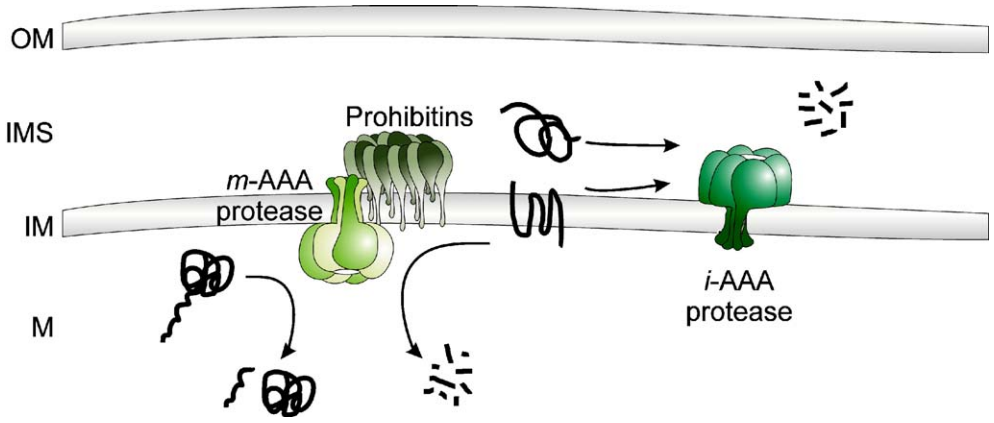

Fig. 3. AAA proteases in the inner membrane of mitochondria. AAA proteases mediate the complete degradation of inner membrane proteins to peptides but can also activate mitochondrial proteins by proteolytic processing. The later activity has been demonstrated for yeast and murine *m*-AAA proteases. *m*-AAA protease assemble with the prohibitin complex in the inner membrane which appears to play a negative regulatory role for proteolysis. Phenotypic analyses in various organisms and genetic evidence in yeast suggest a function of AAA proteases and prohibitins for the maintenance of mitochondrial morphology, but proteolytic substrates of AAA proteases explaining these observations have not been identified. IM, inner membrane; IMS, inter membrane space; M, matrix; and OM, outer membrane.

protease active in the *i*ntermembrane space and *m*-AAA protease in the *m*atrix. Whereas the *i*-AAA protease is a homo-oligomeric complex of Yme1 subunits, the hetero-oligomeric *m*-AAA protease is composed of Yta10 and Yta12 subunits in yeast and Afg3l2 and paraplegin subunits in human. Both AAA proteases exert overlapping substrate specificities and degrade misfolded or non-assembled inner membrane proteins. Moreover, the *m*-AAA protease mediates processing of Ccp1 and importantly also of the ribosomal subunit MrpL32 and thereby controls ribosome assembly and protein synthesis within mitochondria [22,59]. AAA proteases thus have dual activities and mediate both complete degradation as well as proteolytic activation of mitochondrial proteins.

Mitochondrial fragmentation was observed in the absence of the yeast *i*-AAA protease suggesting a function of this protease for mitochondrial dynamics [60]. Mutations in the regulatory proteasomal subunit *ytn1*/*rpt3* suppress the mitochondrial morphology defects caused by the absence of the mitochondrial protease Yme1 [60]. However, the molecular basis of these observations is still obscure. Moreover, they do not appear to reflect a conserved function of the *i*-AAA protease, as morphology defects were not observed upon the loss of the orthologous protease in *Neurospora crassa* [61].

Inactivation of the yeast *m*-AAA proteases leads to respiratory deficiencies in yeast which can be rationalized by the impaired mitochondrial translation in these cells [11]. Mutations in the mammalian *m*-AAA protease subunit paraplegin are causative for cell-specific axonal degeneration in an autosomal recessive form of hereditary spastic paraplegia (HSP) [11]. Notably, mitochondrial abnormalities in synaptic terminals are the first pathogenic defects detected in a paraplegin-deficient mouse model of HSP [62]. Mitochondria are first hypertrophic, and then become enlarged, often reaching huge dimensions, and ultimately the cristae appear swollen and disorganized. The reason for these abnormalities is still mysterious but it is conceivable that they reflect deficiencies in the proteolysis of mitochondrial proteins regulating the morphology of the organelle.

A role of the *m*-AAA protease for mitochondrial dynamics is, at least indirectly, supported by morphology defects associated with the absence of prohibitins in *Caenorhabditis elegans* [63]. Prohibitins constitute large, ring-like complexes in the inner membrane of mitochondria which are composed of highly conserved and ubiquitous Phb1 and Phb2 subunits [64]. These complexes were found to assemble with *m*-AAA proteases both in yeast [65] and mammals (M. Metodiev and T.L., unpublished observations). The function of prohibitins on the molecular level is, however, only poorly understood [66,67]. Deletion of *PHB1* and *PHB2* does not cause apparent growth phenotypes in yeast, but results in an accelerated degradation by the *m*-AAA protease, suggesting a regulatory role during membrane protein turnover [65]. Interestingly, prohibitins are synthetic lethal with Mmm1, Mdm10 and Mdm12, essential components of the mitochondrial inheritance machinery [68–71]. A role of prohibitins for the maintenance of mitochondrial ultrastructure therefore appears to be conceivable. However, alternative scenarios must also be considered, as Mdm10 has recently been linked to the insertion of outer membrane proteins [72] (see review by Stojanovski et al., in this issue).

HtrA2/Omi is yet another protease localised in the inner mitochondrial membrane [73]. During apoptosis, this serine protease is released to the cytosol where it binds and antagonises inhibitor of apoptosis (IAP) proteins (ref). Moreover, the HtrA2 bacterial homologue, DegS, rather than promoting cell death serves protein quality control functions in stress protection [74]. DegS is a sensor of unproperly exposed domains of misfolded proteins [75]. Consequently, it releases the σ^E transcriptional stress activator from an inactive inner membrane associated form to a soluble active form in the bacterial periplasm. Consistently, in mammalian cells, a proteolytic inactive HtrA2 protein (in the *mnd2* mouse model) results in impaired cell survival [76,77]. Moreover, dysfunction of this protease has been linked to Parkinson's disease and neurodegeneration [78,79]. Interestingly, an abnormal mitochondrial morphology was observed upon inactivation of HtrA2 suggesting a role of this peptidase also for mitochondrial dynamics [80].

6. Concluding remarks

Proteolytic processes have now been recognized as playing crucial roles for mitochondrial dynamics, in particular for the regulation of the mitochondrial fusion machinery in the outer membrane. Novel functional links between mitochondrial proteases, the UPS and the morphology of the organelles are emerging that we are just beginning to understand. Further analysis of proteases involved in mitochondrial morphology, e.g., through the identification of their targets will almost certainly provide exciting new insights into the regulation of mitochondrial dynamics.

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